

Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak[☆]

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Abstract

During 2002–2003, exotic Newcastle disease (END) virus caused a major outbreak among commercial and backyard poultry in southern California and adjacent states. The outbreak raised concerns regarding the protective immunity of commercially available vaccines for prevention and control of this virus in poultry. We sought to determine if existing commercial live and inactivated Newcastle disease virus (NDV) vaccines could provide protection against the 2002–2003 END virus, and whether current commercial NDV-vaccination programs for broiler-breeders (BB) and broilers (Br) would protect against END-challenge. In the first experiment, birds received a single dose of either inactivated or live B₁-type vaccine at 2 weeks-of-age and were challenged 2 weeks post-vaccination with a lethal dose of END. In the second experiment, a high ($10^{6.9}$ EID₅₀/bird) or low ($10^{3.9}$ EID₅₀/bird) dose of live B₁ was applied to 8-week-old chickens, followed by lethal END challenge. In the third experiment, NDV field-vaccinated commercial BB (65 weeks-of-age) and Br (36 days-of-age) were challenged against END virus. Results indicated that both the live and inactivated vaccines protected against morbidity and mortality and significantly reduced the incidence and viral titers shed from chickens in comparison with sham controls, but did not prevent infection and virus shedding. In addition, both doses of live vaccine protected birds and significantly decreased the number of birds shedding virus. All unvaccinated control chickens challenged with END died within 6 days post-challenge (pc). Protection from disease correlated with the presence of antibody titers (determined by enzyme-linked immunosorbent assay (ELISA) or hemagglutination inhibition (HI)) at day of challenge. Commercial BB were protected from disease and exhibited low incidence and titer of challenge virus shed. In contrast, commercial Br exhibited 66% mortality and shed significantly more virus than the BB birds. These results underscore the need to develop new NDV vaccines and vaccine strategies for use during outbreak situations to protect birds from both disease and infection to reduce virus shedding.

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1. Introduction

Newcastle disease virus (NDV) is classified as member in the *Avulavirus* genus, within the Paramyxoviridae family. NDV isolates have been classified as lentogenic (low), mesogenic (intermediate) or velogenic (highly virulent) depending on the severity of disease produced by the isolate in chickens [1]. The occurrence of highly virulent NDV infections are recognized as a notifiable disease reportable to the Office of International Epizootics [2]. Velogenic NDV isolates have

[☆] Proprietary or brand names used are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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entered the US via illegal importation of psittacine birds [3–5] and were the causal agent of the last major outbreak in the US in southern California region of the US during 1971–1973 [6,7]. This outbreak of velogenic NDV, also referred to in the US as exotic Newcastle disease (END), resulted in destruction of approximately 12 million birds at a cost of \$56 million. More recent outbreaks of velogenic NDV have been from turkeys in North Dakota during 1992 [8], cormorants in the north-central US [9,10], and game chickens in California during 1998 [11]. Thus, the threat of virulent NDV to commercial US poultry operations is constant.

During May 2002, END virus (ENDV) was isolated from ring neck pheasants in northern California, which preceded diagnosis of ENDV from back yard game chickens in southern California (Los Angeles county) during October 2002 [12]. ENDV was subsequently isolated from commercial poultry in December 2002, and determined to contain nucleotide sequence similarity at the fusion protein cleavage site with the pheasant isolate [13–15]. The first END quarantine zone was imposed in California during November 2002. However, more than 19,000 premises were later quarantined in five states, including California, Nevada, Arizona, Texas and New Mexico. The last positive isolation from commercial poultry was made on 26 March 2003, and the outbreak was deemed eradicated with the last quarantine lifted during September 2003. More than 3 million birds, including approximately 150,000 backyard flocks and 806 commercial sites, were depopulated. Cost of the outbreak is estimated to be in excess of \$200 million.

Current vaccination programs for NDV include the use of low-virulent, live-virus and inactivated vaccines designed to control against endemic, low virulence field strains. The goal of current vaccination procedures is to induce protective immunity while producing a minimal antagonistic response in the bird. For the poultry producer, this decreases economic losses at harvest. Although the efficacy of currently available NDV vaccines against velogenic NDV is widely accepted [16–21], the recent outbreak of END in California underscores the need for continued evaluation of NDV vaccines and vaccination programs. For END outbreak situations, reducing the shed of virus from infected birds is also critical to controlling spread of disease. The objectives of the present study were to extend the knowledge of protection against US ENDV by live and inactivated NDV vaccines, shedding and clearance following virus challenge, and determine immunity of commercially vaccinated birds to a lethal challenge with a California 2002 ENDV isolate.

2. Materials and methods

2.1. Chickens

Mixed sex, specific pathogen-free (SPF) White Plymouth Rock or Leghorn chickens were obtained from the Southeast Poultry Research Laboratory flocks. Birds in experiment I

were held at BSL 2 for vaccination and moved to BSL 3 Ag for challenge [22]. Birds in experiment II were vaccinated and challenged in BSL 3 Ag facilities. Prevacination sera were taken from ten percent of SPF birds prior to group randomization in experiments I and II. Commercial birds, Hubbard Hi Y broiler-breeders (BB) at 64.5 weeks-of-age and broilers (Br) at 36 days-of-age, were received from a local commercial poultry producer. Unchallenged commercial birds were housed in BSL 2 while challenged birds were kept in BSL 3 Ag. All birds were maintained in either Horsfall isolation units or brooder cages with feed and water ad libitum.

2.2. Viruses

Lentogenic NDV vaccine viruses utilized during this study included commercial type B₁ strain B₁ and LaSota (Lohmann Animal Health International (LAHI), Gainesville, GA), Newhatch-C2[®] (Intervet Inc., Millsboro, DE) as well as reference strain B₁ (chicken/US/B1/48). A velogenic strain of END, California 2002 (CA02; game chicken/US(CA)/S0212676/02), was used for all challenge experiments. This isolate was responsible for a recent epizootic outbreak in the southwestern United States recovered from a game bird in California during October 2002 [12]. NDV was propagated and titrated in 9–11-day-old SPF chicken embryos via the chorioallantoic sac route.

2.3. Experimental design

The initial experimentation was designed to assess protection of chickens receiving a single dose of a commercially available inactivated or live NDV B₁ vaccine against challenge from CA02 and determine viral shedding. Subsequently, various doses of live NDV B₁ vaccine were investigated for protection from CA02 challenge. Finally, commercial birds from Georgia that had received routine NDV field vaccination were challenged with CA02 to determine if current industry NDV vaccine strategies would protect against the introduction of this virus to chickens.

2.3.1. Experiment I

Forty-one days-of-age SPF White Rock chickens were arbitrarily divided into four groups of 10 birds. Birds in groups 1 and 2 received 100 μ l of phosphate-buffered saline (PBS; pH 7.4) via intranasal (IN; 50 μ l) and eye drop (ED; 50 μ l) routes at 14 days-of-age. Birds in group 3 received a commercial live-virus B₁B₁ vaccine (LAHI) via ED and IN route according to the manufacturer's recommendations at 14 days-of-age. Birds in group 4 received 100 μ l of inactivated oil-emulsion B₁B₁ vaccine (LAHI) injected subcutaneously in the neck, according to the manufacturers recommendations at 14 days-of-age. Two weeks post-vaccination (day 28), birds in groups 2, 3 and 4 were challenged via ED and IN route with 10^{5.9} embryo infectious dose 50 (EID₅₀)/bird CA02. Unchallenged birds were sham-challenged with 100 μ l PBS via ED/IN route. Following challenge, birds were monitored

daily for overt clinical signs of disease (edema, muscular tremors, torticollis, and paralysis of wings and legs) and mortality. Chickens displaying severe clinical signs of disease were euthanized by overdose of sodium pentobarbital. Serum samples were taken by wing bleed at 0, 7 and 14 days post-challenge (pc). Oropharyngeal and cloacal swabs were collected into 2 ml brain-heart infusion (BHI) broth with antibiotics (1000 units/ml penicillin G, 200 µg/ml gentamicin sulfate, and 4 µg/ml amphotericin B; Sigma Chemical Company, St. Louis, MO) from each bird on 0, 2, 4, 6 and 14 days pc for virus isolation. END-positive swabs were diluted and titrated as described below to determine viral load.

2.3.2. Experiment II

Thirty-two 8-week-old SPF Leghorn chickens were arbitrarily divided into four groups of eight birds. Birds in groups 1 and 2 received 100 µl of PBS. Birds in group 3 received vaccination with a low dose of reference strain B₁ ($10^{3.9}$ EID₅₀/bird). Birds in group 4 received a high dose of reference strain B₁ ($10^{6.9}$ EID₅₀/bird). All vaccines were applied via ED/IN route as described above. At 10 weeks-of-age, birds in groups 2, 3 and 4 were challenged via ED and IN route with $10^{5.9}$ EID₅₀/bird CA02. Birds in group 1 were kept as unchallenged controls. Following challenge, birds were monitored daily for clinical signs of disease and mortality, with serum and swabs processed as described above.

2.3.3. Experiment III

Comparison of protective immunity to CA02 following challenge of field vaccinated broiler-breeders (breeder hens) and broilers from a commercial poultry grower in North Georgia. Prior to being housed at SEPRL, the broiler-breeders and broilers received numerous vaccinations against other infectious agents, including, Marek's disease virus, infectious bronchitis virus and infectious bursal disease virus. Thirteen Hubbard Hi Y layer hens at 65 weeks-of-age had received the following NDV vaccination schedule administered by the grower: B₁ at 13 days-of-age, a high pass LaSota at 5 weeks-of-age, a low pass LaSota at 8 and 16 weeks-of-age, and B₁ at 22, 35, 45, and 55 weeks-of-age. All vaccinations were with live virus given at full dose via drinking water. Fifteen broilers at 36 days-of-age received vaccination at day-of-hatch and 17 days-of-age with a newly available, highly attenuated NDV vaccine (Newhatch-C2[®]), in a spray cabinet and coarse spray in the field, respectively. Ten broiler-breeders and 12 broilers were challenged via ED and IN route with $10^{5.9}$ EID₅₀/bird CA02. As a challenge-control, five SPF White Rock chickens (6 weeks old) were challenged as described above. Three control broiler-breeders and broilers were sham-challenged with PBS. Birds were monitored daily for clinical signs and mortality, with serum and swabs processed as described above.

2.4. NDV enzyme-linked immunosorbent assay (ELISA)

A commercial ELISA test kit (Flockcheck[™] IDEXX Laboratories Inc., Westbrook, ME) was used to test serum and

yolk for antibodies against NDV [24]. Chicken serum samples were diluted 1:500 and incubated in 96-well microtiter plates containing NDV antigen. The ELISA was performed according to the manufacturers' recommendations.

2.5. Hemagglutination (HA) and hemagglutination inhibition (HI) assays

The HA and HI tests were performed by standard microtiter plate methods. The HI tests were performed as previously described, with 4 HA units per well [23].

2.6. Virus isolation and titration

Virus isolation procedures in embryonated chicken eggs followed standard protocols [25]. Virus titers were calculated following inoculation of 10-fold dilutions into 9- or 10-day-old embryonated chicken egg as previously described [23].

2.7. Statistical analysis

Data were analyzed with a statistical software program (SigmaStat 2.0.3, SPSS Inc., Chicago, IL). Analysis of variance using pairwise comparisons with Duncan's and Tukey's method was used to compare ELISA mean values and virus titers, respectively. Frequencies of virus isolation were analyzed for significance by Fisher's exact test. All tests were performed with a 5% level of significance.

3. Results

3.1. Experiment I: protection of SPF birds following vaccination with commercial NDV vaccines

No clinical signs of Newcastle disease were observed in any birds prior to challenge, although one bird in the unchallenged control group died prior to sham-challenge. Protection from END challenge was determined by absence of clinical signs during the 14-days pc observation period. Birds in the unchallenged control group had no clinical signs during the course of the experiment (data not shown). All birds in the sham-vaccinated group (control) displayed conjunctivitis and severe depression from day 2 to 4 pc and 100% mortality was observed at 5 days pc (data not shown). In contrast, no clinical signs or mortality was observed in birds receiving either inactivated (B₁ SQ) or live (B₁ Live) vaccine.

All prevaccination sera tested negative to NDV by both ELISA and HI testing. Antibodies to NDV were detected using ELISA and HI testing on 0, 7 and 14 days pc. As expected, non-vaccinated non-challenged birds (PBS-NC) did not contain positive antibody titers to NDV on any day tested (Table 1). Likewise, unvaccinated END-challenged birds (PBS-C) did not display positive NDV titers prior to challenge. Birds receiving one dose of a commercial live B₁ vaccine 2 weeks prior to challenge exhibited positive ELISA

Table 1

Serum antibody response in experiment I following vaccination of SPF chickens (at 14 days-of-age) with commercial B₁B₁ live- or inactivated virus vaccine and challenge (at 28 days-of-age) with CA02

Group ^a	n	Test	Post-challenge sample ^c		
			Day 0	Day 7	Day 14
PBS-NC	9	ELISA ^b	52 ^a (34)	15 ^a (4)	283 ^a (155)
		HI ^c	2	3.1	3.1
PBS-C	10	ELISA	54 ^a (76)	NS ^d	NS
		HI	2	NS	NS
Live B ₁ -C	10	ELISA	688 ^a (174)	5149 ^b (876)	9143 ^b (1040)
		HI	6.2	>9	>9
Inactivated B ₁ -C	10	ELISA	4045 ^b (1057)	12243 ^c (1255)	15961 ^c (1299)
		HI	>9	>9	>9

^a NC: not challenged, C: challenged with 10^{5.9} EID₅₀ CA02 NDV, n: number of chickens per group.

^b Results are expressed as mean and standard error of the mean in parenthesis. Titers >396 are considered positive.

^c Geometric mean titer expressed as reciprocal log₂. Titers ≥4 considered positive.

^d NS: no survivors.

^e Means within a column with different superscript letters are significantly different ($P < 0.05$).

(688) and HI (6.2) titers to NDV, which increased throughout the course of the challenge. Antibody levels in this group were significantly higher than those in the non-vaccinated group on days 7 and 14 pc. Birds vaccinated subcutaneously with a single dose of inactivated B₁ also displayed positive NDV ELISA (4045) and HI (≥9) titers. The antibody response to NDV was significantly higher in the groups receiving inactivated vaccine than live vaccine on all days tested.

Virus isolation results from swabs taken on days 0, 2, 4, 6 and 14 pc in experiment I are presented in Table 2. END virus was isolated from each unvaccinated END-challenged bird at day 2 and 4 pc from both oral and cloacal swabs. No virus was isolated from any bird in the unchallenged group. Oral swabs taken from inactivated B₁ vaccinated chickens exhibited positive isolations from 90 and 70% of birds on days 2 and 4 pc, respectively. Birds vaccinated with live B₁ displayed positive virus isolations with oral swabs from 30, 30 and 10% of birds on days 2, 4 and 6 pc, respectively. Virus isolation from cloacal swabs first appeared at 4 days pc in 60 and 30% of the birds receiving inactivated or live B₁ vaccine, respectively. No virus isolations were observed from oral or cloacal swabs at day 14 pc in any group.

High titers of END-virus were isolated from END-positive control SPF birds from both oral and cloacal swabs (Fig. 1A

and B). Titers in this group increased following END-challenge, with greater than 10⁶ EID₅₀/ml recovered from both swabs at day 4 pc. Birds receiving inactivated B₁ displayed END titers of approximately 5 × 10³ EID₅₀/ml 2 days pc from oral swabs that decreased at day 4 pc. Viral titers recovered from cloacal swabs were highest at 4 days pc (2 × 10² EID₅₀/ml). Birds receiving B₁ live vaccine displayed the lowest levels of virus shed on days 2 and 4 pc (≤1.2 × 10¹ EID₅₀/ml) from oral swabs. Virus recovered from cloacal swabs was determined to be <1.0 × 10¹ EID₅₀/ml on days 4 and 6 pc from birds in this group.

3.2. Experiment II: comparison of protective immunity between low (10^{3.9} EID₅₀/bird) and high (10^{6.9} EID₅₀/bird) doses of NDV B₁ vaccine

No overt clinical signs of Newcastle disease were observed in any chickens prior to challenge. All birds in the non-challenged control group remained normal during the course of the experiment (data not shown). The sham-vaccinated END-challenged birds displayed conjunctivitis, severe depression, inactivity and diarrhea from days 2 to 4 pc with 100% mortality on day 6 pc (data not shown). Neither clinical signs of disease nor mortality were observed in groups

Table 2

CA02 virus isolation in experiment I following vaccination with either live or inactivated B₁ vaccine

Group ^a	Post-challenge sample ^b (no. positive/total)									
	Day 0		Day 2		Day 4		Day 6		Day 14	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
PBS-NC	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a	0/9 ^a
PBS-C	0/10 ^a	0/10 ^a	10/10 ^b	10/10 ^b	10/10 ^b	10/10 ^b	NS ^c	NS	NS	NS
Inactivated B ₁ -C	0/10 ^a	0/10 ^a	9/10 ^b	0/10 ^a	7/10 ^{b,c}	6/10 ^{b,c}	0/10 ^a	1/10 ^a	0/10 ^a	0/10 ^a
Live B ₁ -C	0/10 ^a	0/10 ^a	3/10 ^a	0/10 ^a	3/10 ^{a,c}	3/10 ^{a,c}	1/10 ^a	1/10 ^a	0/10 ^a	0/10 ^a

^a NC: not challenged, C: challenged with 10^{5.9} EID₅₀ CA02 NDV.

^b Different superscript letters denote significant difference ($P < 0.05$) between groups with the column.

^c NS: no survivors.

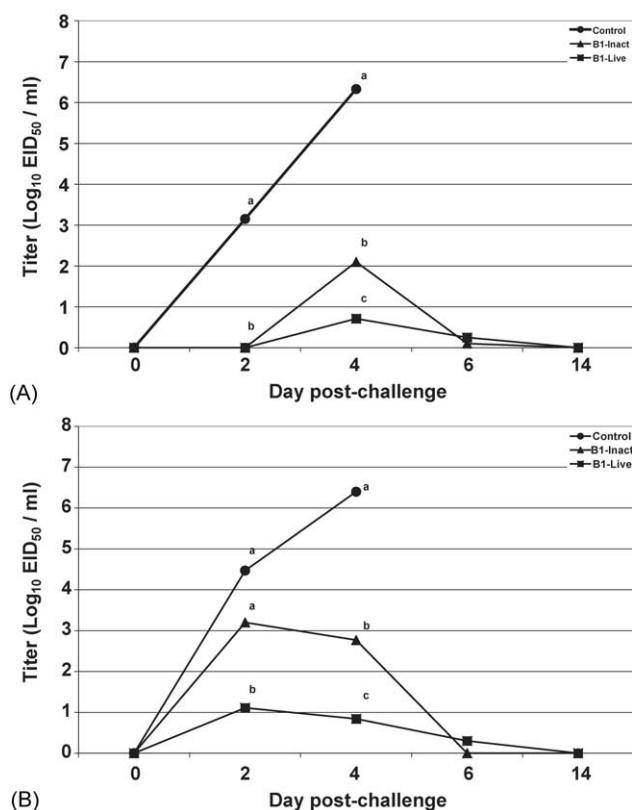


Fig. 1. Reduction of virus shed following vaccination with NDV B₁ vaccines in experiment I. Chickens were vaccinated with either a commercial live or inactivated B₁ vaccine by eye drop/intranasal or subcutaneous route, respectively, at 14 days-of-age. Birds were challenged at 28 days-of-age with $10^{5.9}$ EID₅₀/bird CA02 via eye drop/intranasal route. Oral (A) and cloacal (B) swabs were sampled on the days indicated.

of birds vaccinated with either a low or high dose of live B₁ vaccine during the course of the experiment.

All prevaccination sera tested negative for antibodies to NDV by both ELISA and HI. Non-vaccinated non-challenged birds did not exhibit positive ELISA or HI antibody titers to NDV on days 0 or 14 pc (Table 3). Unvaccinated END-challenged birds did not display positive antibody titers prior to challenge. Birds receiving a low dose of live B₁ (Low B₁-C) for vaccination exhibited positive ELISA (7038) and HI (8) titers to NDV at day 0 which increased following END-

Table 3

Serum antibody response in experiment II following vaccination of SPF chickens with either high or low dose reference B₁ live virus vaccine (at 8 weeks-of-age) and challenge (at 10 weeks-of-age) with CA02

Group ^a	n	Test	Post-challenge sample ^e	
			Day 0	Day 14
PBS-NC	8	ELISA ^b HI ^c	63 ^a (16) 1	26 ^a (8) 1
PBS-C	8	ELISA HI	61 ^a (17) 0.8	NS ^d NS
Low B ₁ -C	8	ELISA HI	7038 ^b (1378) 8	12902 ^b (2001) 8.3
High B ₁ -C	8	ELISA HI	13904 ^c (2146) 8	15240 ^b (1730) 8.3

^a NC: not challenged, C: challenged with $10^{5.9}$ EID₅₀ CA02 NDV. Low B₁ vaccinated birds received $10^{3.9}$ EID₅₀/bird. High B₁ vaccinated birds received $10^{6.9}$ EID₅₀/bird. n: number of chickens per group.

^b Results are expressed as mean and standard error of the mean in parenthesis. Titers >396 are considered positive.

^c Geometric mean titer expressed as reciprocal log₂. Titer ≥4 are considered positive.

^d NS: no survivors.

^e Means within a column with different superscript letters are significantly different ($P < 0.05$).

challenge. Antibody levels in this group were significantly higher than those in both non-vaccinated groups on day 0 and the non-challenged group on day 14 pc. Birds vaccinated with a single high dose of live B₁ (High B₁-C) also displayed positive NDV ELISA (13904) and HI (8) titers. The antibody response to NDV was significantly higher than all others on day 0, and significantly higher than controls on day 14 pc.

Virus isolation results from swabs taken on days 0, 2, 4, 6 and 14 pc in experiment II are presented in Table 4. No virus was isolated prior to challenge or in the unchallenged group during the course of the study. END virus was isolated from all unvaccinated END-challenged birds at days 2 and 4 pc from both oral and cloacal swabs. No virus positive swabs were observed in cloacal samples from either the low or high dose of B₁ vaccinated groups on any day tested. Oral swabs taken from low dose B₁ vaccinated chickens exhibited positive isolations in 25, 13 and 13% of birds on days 2, 4 and 6 pc, respectively. Birds vaccinated with high dose B₁

Table 4

CA02 virus isolation in experiment II following vaccination with either a low or high dose of live reference B₁

Group ^a	Post-challenge sample ^b (no. positive/total)									
	Day 0		Day 2		Day 4		Day 6		Day 14	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
PBS-NC	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a
PBS-C	0/8 ^a	0/8 ^a	8/8 ^b	8/8 ^b	8/8 ^b	8/8 ^b	NS ^c	NS	NS	NS
Low B ₁ -C	0/8 ^a	0/8 ^a	2/8 ^a	0/8 ^a	1/8 ^a	0/8 ^a	1/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a
High B ₁ -C	0/8 ^a	0/8 ^a	3/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a	0/8 ^a

^a NC: not challenged, C: challenged with $10^{5.9}$ EID₅₀ CA02 NDV.

^b Different superscript letters denote significant difference ($P < 0.05$) between groups with the column.

^c NS: no survivors.

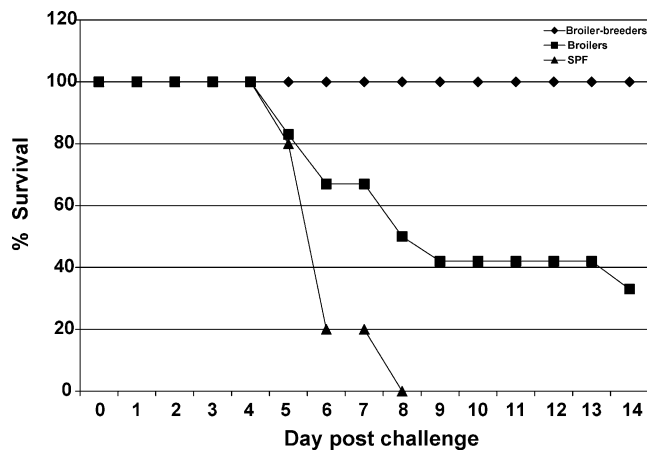


Fig. 2. Survival of commercial broiler-breeders and broilers receiving field vaccination against NDV and challenged with CA02. Chickens were infected via eye drop/intranasal route with $10^{5.9}$ EID₅₀/bird CA02 and mortality observed over a 2-week period.

only exhibited positive virus isolations from oral swabs on day 2 pc in 38% of birds. No virus isolations were observed in any group from swabs taken at day 14 pc.

3.3. Experiment III: challenge of commercial broiler-breeders and broilers

No overt clinical signs of Newcastle disease were observed in any group prior to challenge. The non-challenged broiler-breeders and broilers did not exhibit any clinical signs of Newcastle disease during the course of the study. Eighty percent of sham-vaccinated END-challenged SPF birds displayed conjunctivitis within 48 h of challenge. All birds in this group (SPF) displayed bilateral conjunctivitis and severe depression from days 3 to 4 pc, and 40% mortality was observed on day 5 pc (Fig. 2). Remaining birds displayed diarrhea and inactivity from days 5 to 7, and 100% mortality was observed on day 8 pc. In contrast, no clinical signs of Newcastle disease or mortality was observed in the group of END-challenged broiler-breeders throughout the course of the experiment. Fifty percent (6/12) of END-challenged broilers displayed conjunctivitis on day 2 pc. By day 3 pc 58% (7/12) of broilers displayed bilateral conjunctivitis. On day 4 pc, tremors and diarrhea was observed in two birds. On day 5 pc, mortality was observed in two birds and seven birds appeared lethargic. The remaining 3 birds appeared normal and remained healthy throughout the course of the challenge. Late mortality occurred in birds displaying lethargy at earlier time points.

As expected, non-vaccinated END-challenged SPF birds were NDV antibody negative prior to challenge (Table 5). Unchallenged broiler-breeders (Broiler-breeders-NC) had positive NDV titers by both ELISA (720) and HI (≥ 9) on day of challenge. However, in the absence of END-challenge, the ELISA (242) and HI (7) titers from these birds dropped by day 14 pc. Both ELISA (684) and HI (8.6) titers from the broiler-

Table 5

Serum antibody response of commercial broiler-breeders (66 weeks-of-age) and broilers (44 days-of-age) in experiment III following challenge with CA02

Group ^a	n	Test	Post-challenge sample ^d	
			Day 0	Day 14
Broiler-breeders-NC	3	ELISA ^b HI ^c	720 ^a (210) >9	242 ^a (70) 7
Broiler-breeders-C	10	ELISA HI	684 ^a (217) 8.6	2827 ^b (403) >9
Broilers-NC	3	ELISA HI	15 ^{a,b} (6) 4.3	11 ^a (7) 3
Broilers-C	12	ELISA HI	15 ^{a,b} (6) 3.3	3787 ^b (524) ^f >9
Control SPF-C	5	ELISA HI	5 ^b (2) 2.6	NS ^e NS

^a NC: groups of birds not challenged, C: groups of birds challenged with $10^{5.9}$ EID₅₀ CA02 NDV. n: number of chickens per group.

^b Results are expressed as mean and standard error of the mean in parenthesis. Titers >396 are considered positive.

^c Geometric mean titer expressed as reciprocal log₂. Titers ≥ 4 are considered positive.

^d Means within a column with different superscript letters are significantly different ($P < 0.05$).

^e NS: no survivors.

^f Mean titer of remaining four birds.

breeders that received END-challenge (Broiler-breeders-C) increased during challenge to 2827 and ≥ 9 , respectively. As a group, the commercial broilers (Broilers-NC or Broilers-C) did not display positive ELISA titers to NDV prior to challenge. However, the three of the four surviving birds displayed positive HI titers (5) on day of challenge. Fourteen days after END-challenge, ELISA (3787) and HI (≥ 9) titers had increased in the broiler survivors.

Virus isolation results from swabs taken on days 0, 2, 4, 6, 9 and 14 pc in experiment III are presented in Table 6. No virus was isolated from any bird in the unchallenged groups. Challenge virus was reisolated in oral and cloacal swabs from 3 control SPF birds at day 2 pc and all birds at 4 days pc. Both swabs were positive for END virus from the lone surviving bird at day 6 pc. Few of the broiler-breeders challenged shed END virus. Oral swabs taken from this group were only positive on days 4 and 6 pc in $\leq 20\%$ of birds. Cloacal swabs were positive on days 4, 6, and 9 in 20, 30 and 10% of birds, respectively. END-challenged commercial broilers displayed a higher rate of virus shed than the broiler-breeders. Virus was reisolated from 92, 100 and 63% of oral swabs from challenged broilers (Broilers-C) on days 2, 4 and 6 pc, respectively. Cloacal swabs were positive in 58, 100, 25 and 40% of birds on days 2, 4, 6, and 9 pc, respectively. Virus isolation results at day 14 pc of all samples were negative from all birds.

High titers of challenge virus could be detected from both oral and cloacal swabs in the control SPF group, with the highest titers ($>10^5$ EID₅₀/ml) recovered from days 6 and 4 pc, respectively (Fig. 3). Commercial broiler-breeders had reduced

Table 6

CA02 virus isolation in experiment III following challenge of commercial broiler-breeders (BB) and broilers (Br)

Group ^a	Post-challenge sample ^b											
	Day 0		Day 2		Day 4		Day 6		Day 9		Day 14	
	O	C	O	C	O	C	O	C	O	C	O	C
BB-NC	0/3 ^a	0/3 ^a	0/3 ^{a,b}	0/3 ^{a,b}	0/3 ^a	0/3 ^a	0/3 ^{a,b}	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a
BB-C	0/10 ^a	0/10 ^a	0/10 ^a	0/10 ^a	2/10 ^a	2/10 ^a	1/10 ^a	3/10 ^a	0/10 ^a	1/10 ^a	0/10 ^a	0/10 ^a
Br-NC	0/3 ^a	0/3 ^a	0/3 ^{a,b}	0/3 ^{a,b}	0/3 ^a	0/3 ^a	0/3 ^{a,b}	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a
Br-C	0/12 ^a	0/12 ^a	11/12 ^c	7/12 ^b	12/12 ^b	12/12 ^b	5/8 ^b	2/8 ^a	0/5 ^a	2/5 ^a	0/4 ^a	0/4 ^a
Control SPF-C	0/5 ^a	0/5 ^a	3/5 ^{b,c}	3/5 ^b	5/5 ^b	5/5 ^b	1/1 ^{a,b}	1/1 ^a	NS ^c	NS	NS	NS

^a NC: birds not challenged, C: groups of birds challenged with $10^{5.9}$ EID₅₀ CA02 NDV.^b Total number of positive/total number in each group. O: oral swab, C: cloacal swab. Different superscript letters denote significant difference ($P < 0.05$) between groups with the column.^c NS: no survivors.

titers of END-challenge virus in both the oral and cloacal swabs on all days tested in comparison with the control SPF and vaccinated-broiler groups. Less than 10^1 EID₅₀/ml challenge virus was recovered from birds in this group throughout the testing period. In contrast, oral swabs recovered from commercial broilers receiving two NDV field vaccinations prior to challenge displayed titers similar to control SPF birds by day 4 pc. However, surviving birds were able to resolve infection rapidly and no titers were observed in oral swabs

after day 6 pc. Cloacal titers of challenge virus peaked on day 4 pc ($10^{3.7}$ EID₅₀/ml).

4. Discussion

The continued outbreaks of velogenic NDV in domestic poultry worldwide emphasize the importance for continued research on vaccine efficacy against newly isolated strains. In October 2002, END virus was isolated from backyard poultry in California and was responsible for a major outbreak that spread to commercial poultry in that region [12]. Information regarding vaccine efficacy against END virus isolates will provide valuable knowledge for the poultry industry when considering vaccine types and vaccination strategies. We demonstrated that commercial inactivated and live B₁ vaccines protected SPF birds against morbidity and mortality from challenge with the highly virulent California 2002 ENDV. All vaccinated birds displayed antibody titers against NDV and a positive correlation was observed between the presence of positive antibody titers (either by ELISA or HI) at day of challenge and protection from disease. The protective role of ELISA and HI antibody titers against NDV has been described [26]. The live B₁ vaccine was superior to the inactivated vaccine in inducing immunity levels that resulted in decreased virus titers shed from the respiratory and intestinal tract recovered on both oral and cloacal swabs. Commercially vaccinated birds displayed varying degrees of protection that also appeared to correlate with the presence of anti-NDV antibody titers. The results extend the findings of prior reports of protection in poultry against velogenic NDV using commercial lentogenic vaccines [19,20,27,28].

Experiment I demonstrated that humoral immunity conferred by single application of commercial B₁ vaccines resulted in increased antibody titers in SPF birds. While these birds were protected from clinical disease, they were not protected against infection. The high titers ($10^{3.2}$ – $10^{6.4}$ EID₅₀/ml) of infectious virus recovered from oral and cloacal swabs in control chickens during the first 4 days after challenge allowed us to evaluate protection in the respiratory and intestinal tract of vaccinated animals. The

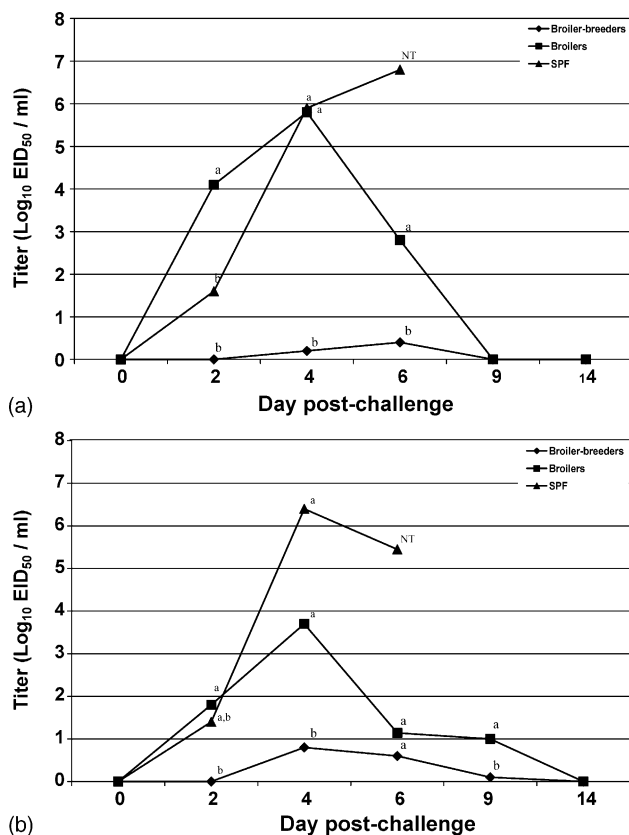


Fig. 3. Comparison of CA02 mean virus titers from oral and cloacal swabs following challenge with CA02 virus in experiment III. Commercial broiler-breeders and broilers received field NDV vaccination and were challenged with $10^{5.9}$ EID₅₀/bird CA02. Oral (A) and cloacal (B) swabs were sampled on the days indicated. NT: not tested.

live virus vaccine significantly reduced both the number of chickens shedding the CA02 virus and the titers shed (>5 log reduction on day 4 pc) from vaccinated chickens compared to controls. The inactivated vaccine significantly reduced the titers shed (>4 log reduction on day 4 pc) from challenged birds compared to controls, however, no significant difference was observed in the incidence of infection between birds in these groups. In addition, administration of the live vaccine resulted in fewer birds shedding virus from the oral and cloacal route compared to birds receiving inactivated vaccines. This is not surprising since a live virus vaccine would be expected to generate antibodies for protection on mucosal surfaces and thus provide better protection against infection [29].

Having established protection with the commercial vaccines, the effect of dose on protection by live B₁ was examined. Chickens in experiment II were vaccinated with either a high or low dose of live B₁ virus. A positive correlation was observed between presence of antibody titers at day-of-challenge and protection from disease. In addition, birds receiving the higher dose of B₁ had significantly higher ELISA antibody titers at day-of-challenge than birds receiving the lower dose, although no difference was observed in HI titers between these groups. Vaccination with either dose resulted in significantly fewer birds shedding virus compared to control-challenged birds. Apparently the vaccine dose of live B₁ had no effect on the incidence of shedding following challenge, as few birds were determined to shed virus throughout the experiment. The last virus isolation was obtained on day 6 post-challenge in the low-dose group of birds, which correlated with the last day of virus isolation obtained from birds receiving live virus vaccine in experiment I. Since diluting vaccines is a routine practice in the field to decrease vaccine reaction, as well as decrease cost per bird for the grower, it was noteworthy that the different doses used in this experiment both protected birds equally. However, dilutions performed in this experiment were based on known virus titers to attain known dosage levels.

Experiment III confirmed that broiler-breeder vaccination programs are effective at protecting commercial birds from END challenge. As observed in our previous experiments, a positive correlation was observed between the presence of antibody titers at challenge and protection from disease. In addition, these birds had low incidence of virus shedding and virus titers (>5 log reduction on day 4 pc) recovered from those birds were significantly lower than either control-challenged birds or commercial broilers. Considering the number of NDV vaccinations given to these birds throughout their life, it was not surprising that these birds were resistant to challenge and had few birds shedding virus.

Experiment III showed that commercial broilers vaccinated at 1 and 17 days-of-age were susceptible to END challenge. Although the geometric mean NDV antibody titers from this group were negative by both ELISA and HI testing at day of challenge, three of the four surviving broilers did display positive HI titers prior to challenge. Also evident in the challenged commercial broilers was the increased inci-

dence of birds shedding virus, titer of virus recovered, and duration of shedding by birds in this group compared to the challenged broiler-breeders.

Failure of this vaccine to induce immunity following two vaccinations of the commercial broilers is confounding. Several factors may have contributed to the poor response observed. First, since maternal antibody can be detected in serum up to 3 weeks-of-age, it is possible that the vaccine virus was neutralized by maternal antibody [30]. Yolk antibodies recovered from eggs produced by the broiler-breeders during the course of challenge were determined to contain high ELISA and HI antibody titers, which would be passed on to progeny broilers. In addition, it is unknown if the birds received a full dose of the vaccine, or if the vaccine was properly handled and properly administered in the field. The immune competence of the birds, either by age or presence of immunosuppressive etiologic agents, may have also contributed to the lack of response to the vaccine in these birds. Since the potency of the vaccine was established during product licensure, it is likely that the failure to protect the commercial broilers against challenge was due to confounding factors mentioned above rather than lack of potency of the product.

In these studies, although NDV-vaccinated birds were protected against END, they continued to shed virus in the absence of clinical signs up to 9 days pc. Following an outbreak situation this condition may prevent diagnosis of an infected flock and result in further spread of disease and duration of the outbreak. During the 1971–1974 END outbreak mass application of NDV vaccines were applied to commercial poultry to help improve immunity of the birds and eradicate the disease. However, evaluation of the vaccination program showed that although vaccination reduced mortality in END-infected flocks, it failed to stop the spread of disease, regardless of the vaccine, route or frequency of use [31]. Parental immunity contributed to problems associated with vaccination of young chicks, an observation speculated on in these studies given the high yolk antibody titers recovered from eggs and the poor broiler-vaccine performance. The use of mass vaccination also interfered with detection and diagnosis of infected flocks. It was recommended that mass vaccination of poultry not be used for future outbreaks unless better vaccines become available [31]. Although 30 years have passed since that outbreak, the poultry industry still relies heavily on vaccines available during that time. While these vaccines help protect against morbidity and mortality from low virulent field strains, it is evident that testing both SPF and commercially vaccinated birds is critical to measuring vaccine protection and designing vaccination strategies for future outbreaks. Recent advances and technologies have resulted in the formulation of numerous new generation NDV vaccines, including DNA [32], virus-vectored [33–35], virosome [36], and recombinant vaccines [37], which have been shown to varying degrees to induce protective immunity against morbidity and mortality. The development of improved vaccines and vaccination strategies to induce protection against infection and

inhibit shed of virus are needed. Presumably, these should target cellular and humoral immunity to inhibit spread of the virus in the bird, as well as respiratory and gut immunity to prevent or decrease duration and level of virus shed from mucosal surfaces. Since new technologies exist for rapid detection and characterization of low virulent vaccine strains from virulent strains, the use of vaccination for outbreak situations would not hinder diagnosis [38]. Although such a vaccine(s) may not be attainable or warranted in the absence of virulent NDV, when the next outbreak occurs vaccination can play a vital role in the control and eradication of END in the US.

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